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Identification of Mesophilic Lactic Acid Bacteria by Using Polymerase Chain Reaction-Amplified Variable Regions of 16S rRNA and Specific DNA Probes

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Specific DNA probes based on variable regions V1 and V3 of 16S rRNA of lactic acid bacteria were designed. These probes were used in hybridization experiments with variable regions amplified by using the polymerase chain reaction. In this way, a rapid and sensitive method was developed for the identification and classification of *Lactococcus* and *Leuconostoc* species.

Lactic acid bacteria (LAB) are of great economic importance for the dairy and other fermented food industries. For both basic research on LAB and their application in industrial food fermentations, reliable and simple methods for identification of such bacteria are required. Because many LAB have similar nutritional and growth requirements, it is very difficult to identify them by classical methods. Therefore, various approaches that use molecular probes have been described (2, 4, 11). Here we report on a combination of sensitive techniques for identification and detection of LAB that is based on polymerase chain reaction (PCR) (13) and specific DNA probing (9).

In recent years, the use of rRNA sequences for identification and phylogenetic analysis has been generally accepted (1, 5). DNA probes based on highly variable rRNA regions have been applied successfully for the identification and detection of microorganisms in soil, intestinal tract, and clinical samples (6, 12, 16). By comparing the published 16S rRNA sequences of *Lactococcus* spp. (3, 14) and *Leuconostoc* spp. (8, 17), we identified the regions containing the highest variability. For the genus *Lactococcus*, described by Schleifer et al. (15), the V1 region (90 bp) contained sufficient sequence variation to enable the design of DNA probes allowing differentiation between the species *Lactococcus lactis*, *L. garvieae*, *L. plantarum*, and *L. raffinolactis* and *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The sequences of the V1 region (90 bp) appeared to be identical in all species analyzed in the genus *Leuconostoc*, but those of the V3 region contained sufficient variation to design DNA probes specific for *Leuconostoc* species (Table 1).

To increase the sensitivity of the procedure, we used PCR amplification of the variable regions with primers based on the conserved flanking sequences (Table 1 and Fig. 1). The PCR amplifications were performed by using a BioMed Thermocycler (BioMed, Amstelstad, Holland). The reactions were carried out in sterile Multimax seal tubes with cap locks (1.5 ml), which contained 50 μl of the following buffer: 10 mM Tris HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, deoxynucleoside triphosphates at 2.5 mM, and 1 U of Taq polymerase. Template DNA (500 to 100 ng) was added after being heated to 95°C to eliminate all protease activity. The amplification was done in 30 cycles by melting the DNA at 93°C for 1 min, annealing at 54°C for 1.5 min, and elongation at 72°C for 2.5 min.

To allow the identification of small amounts of bacteria, a method was developed to isolate DNA from a single colony grown on an agar plate (7). After the colony (±1.5-mm diameter) was suspended in 50 μl of 10 mM Tris HCl buffer (pH 8.0) containing 400 μg of lysozyme and incubation at 37°C, the cells were lysed by adding 50 μl of 10% sodium dodecyl sulfate and 250 μl of buffer. The DNA was precipitated by adding 60 μl of 3 M sodium acetate and 1 ml of 96% ethanol (stored at −20°C). After centrifugation, the DNA pellet was dissolved in 10 mM Tris HCl buffer (pH 8.0) and precipitated a second time by adding 1 ml of isopropanol. The DNA pellet was washed with 70% ethanol and finally dissolved in 50 μl of TE buffer (10 mM Tris HCl [pH 8.0], 1

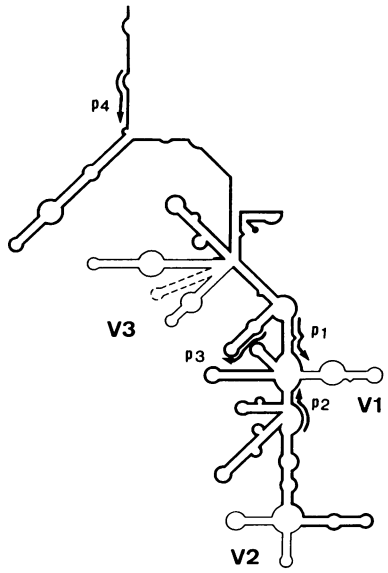

FIG. 1. Representation of the 5′ region of the secondary structure of 16S rRNA (●, 5′ terminus). Conserved areas are drawn in bold lines, and areas that vary in sequence and size are drawn in thin lines (broken lines, structure found only in a few organisms) (10). This part of the 16S rRNA contains the variable regions V1, V2, and V3. The location and direction of the PCR primers used in this study are marked by arrows.

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TABLE 1. List of primers used

Use	Primer or probe[a]	Target DNA (region)	Sequence (5′ to 3′)
PCR	P1 (S)	41–60 (V1)[b]	GCGGCGTGCCTAATACATGC
	P2 (A)	111–130 (V1)[b]	TTCCCCACGCGTTACTCACC
	P3 (S)	361–380 (V3)[b]	GGAATCTTCCACAATGGGCG
	P4 (A)	685–705 (V3)[b]	ATCTACGCATTTCACCGCTAC
DNA probe	PLl_1 (A)	*L. lactis* subsp. *lactis* V1	AGTCGGTACAAGTACCAAC
	PLl_2 (S)	*L. lactis* subsp. *lactis* and *L. lactis* subsp. *hordniae* V1	GCTGAAGGTTGGTACTTGTA
	PLc (A)	*L. lactis* subsp. *cremoris* V1	TTCAAATTGGTGCAAGCACC
	PLp (A)	*L. plantarum* V1	CTACGGTACAAGTACCAGT
	PLg (A)	*L. garvieae* V1	CATAAAAATAGCAAGCTATC
	PLr (A)	*L. raffinolactis* V1	CGGTGAAGCAAGCTTCGGT
	PLC (A)	*Leuconostoc* spp. V1	CACCTTTCGCTGTGGTT
	PLCl (S)	*Leuconostoc lactis* V3	ATGCTAGAATAGGGAATGAT
	PLCm (S)	*Leuconostoc mesenteroides* V3	CAGCTAGAATAGGAAATCAT

[a] S, sense sequence; A, antisense sequence.
[b] *Escherichia coli* numbering is used (10).

mM EDTA). Five microliters of this solution was used for PCR amplification.

After agarose gel electrophoresis, the PCR-amplified fragments were transferred to GeneScreen Plus (Dupont, Boston, Mass.) with a vacuum blotter (Pharmacia, Woerden, Holland). Prehybridization and hybridization were performed in 0.5 M sodium phosphate buffer (pH 7.2) containing 3% sodium dodecyl sulfate and 1% bovine serum albumin. After 30 min of prehybridization at 40°C, the probe, which had been 5′-end labeled with [γ-^{32}P]ATP (Radiochemical Centre, Amersham, England), was added and the incubation was continued for 4 h. The blots were washed with 0.3 M NaCl–0.03 M sodium citrate at 37°C until a clear signal was found and then were exposed to Kodak X-ray films.

Figure 2 shows that it is possible to identify and discriminate various *Lactococcus* strains with DNA probes that are based on the highly variable V1 region. The specificity of these probes was tested on some closely related LAB (Table 2). The PLl_1 probe did not give a signal with *L. lactis* subsp. *hordniae* containing a sequence in the V1 region that differs in only one nucleotide from that of *L. lactis* subsp. *lactis* and its variant *diacetylactis* (data not shown). Exactly the same substitution is found in the V1 region of some *L. lactis* subsp. *lactis* strains (14). By using the PLl_2 probe, these variants could also be detected (Table 2). The sequences of the V1 regions of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* show too many differences to allow the design of a species-specific *L. lactis* probe.

The identification of *Leuconostoc* spp. is shown in Fig. 3. The larger size of its amplified V1 region (Fig. 3A) confirms that *Leuconostoc paramesenteroides* is related to the genus *Lactobacillus* (V1 = 110 bp), as was proposed recently on the basis of 16S rRNA comparison (18). In spite of the fact that the V3 regions of *Leuconostoc lactis* and *Leuconostoc mesenteroides* contained only three nucleotide differences, a good discrimination between these species was found with the *Leuconostoc* probes (Fig. 3). The specificity of these probes was also confirmed (Table 3).

TABLE 2. Strains tested with DNA probes based on the V1 region[a]

Strain	Specificity of probe:						
	PLl_1	PLl_2	PLc	PLp	PLg	PLr	PLC
Lactococcus lactis subsp. *lactis* NCFB 2597	+	+	−	−	−	−	−
L. lactis subsp. *lactis* NCFB 764	+	+	−	−	−	−	−
L. lactis subsp. *lactis* NIZO R5	+	+	−	−	−	−	−
L. lactis subsp. *cremoris* NCFB 1200	−	−	+	−	−	−	−
L. lactis subsp. *cremoris* NCFB 504	−	−	+	−	−	−	−
L. lactis subsp. *cremoris* NIZO HP	−	−	+	−	−	−	−
L. lactis subsp. *lactis* variant *diacetylactis* NCFB 176	+	+	−	−	−	−	−
L. lactis subsp. *hordniae* NCFB 2181	−	+	−	−	−	−	−
L. plantarum NCFB 1869	−	−	−	+	−	−	−
L. garvieae NCFB 2155	−	−	−	−	+	−	−
L. raffinolactis NCFB 617	−	−	−	−	−	+	−
Leuconostoc mesenteroides NCFB 523	−	−	−	−	−	−	+
Leuconostoc lactis NCFB 533	−	−	−	−	−	−	+
Vagococcus fluvialis NCFB 2497	−	−	−	−	−	−	−
Streptococcus mutans ATCC 10449	−	−	−	−	−	−	−
Streptococcus sanguis ATCC 10556	−	−	−	−	−	−	−
Streptococcus thermophilus NIZO St1	−	−	−	−	−	−	−
Enterococcus faecalis LMG 7937	−	−	−	−	−	−	−
Staphylococcus aureus ATCC 14459	−	−	−	−	−	−	−

[a] Designation of probes is according to Table 1.

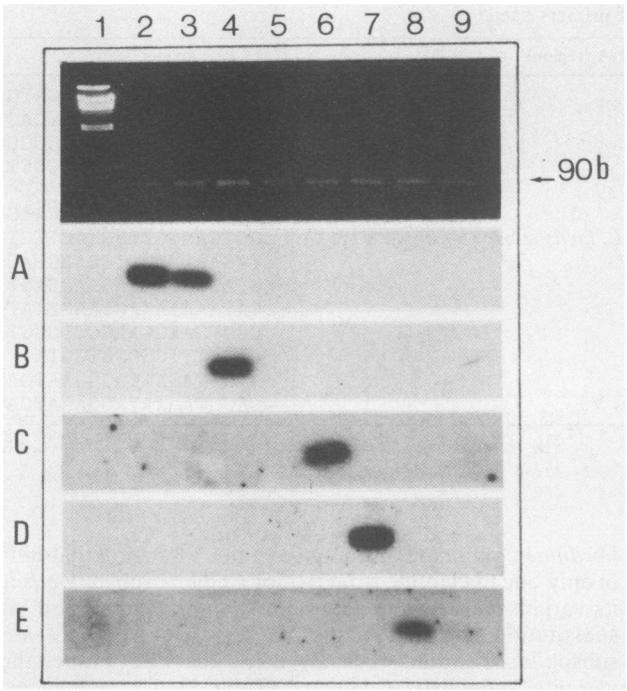

FIG. 2. Identification of *Lactococcus* species and subspecies. The top panel shows an ethidium bromide-stained 1% agarose gel used for separation of the PCR-amplified V1 region of 16S rRNA genes, using primers P1 and P2. Lane 1, lambda DNA digested with *Hin*dIII, used as a negative control for background hybridization; lane 2, *L. lactis* subsp. *lactis* NCBF 2597; lane 3, *L. lactis* subsp. *lactis* variant *diacetylactis* NCBF 176; lane 4, *L. lactis* subsp. *cremoris* NCBF 1200; lane 5, *L. lactis* subsp. *hordniae* NCBF 2181; lane 6, *L. plantarum* NCBF 1869; lane 7, *L. garvieae* NCBF 2155; lane 8, *L. raffinolactis* NCBF 617; lane 9, *Vagococcus fluvialis* NCBF 2497. Gels run in parallel, which contained identical samples, were blotted and hybridized with the PLl_1 (A), the PLc (B), the PLp (C), the PLg (D), or the PLr (E) probe.

The specific DNA probes designed and evaluated in this study allow the identification of small amounts of LAB. The described methods have two major advantages compared with classical identification techniques. First, it is possible to obtain a reliable identification within 1 or 2 days. Second, it is possible to perform a simultaneous identification of a large number of strains with only a small amount of cells, one colony on an agar plate being sufficient. Because of these advantages, the methods are well suited to characterize isolates from starter cultures and environmental samples.

TABLE 3. Strains tested with DNA probes based on the V3 region[a]

Strain	Specificity of probe:	
	PLCl	PLCm
Leuconostoc mesenteroides NCFB 523	−	+
Leuconostoc mesenteroides NIZO 3406	−	+
Leuconostoc mesenteroides NIZO 3411	−	+
Leuconostoc lactis NCFB 533	+	−
Leuconostoc lactis NIZO 6009	+	−
Leuconostoc lactis NIZO 6070	+	−
Leuconostoc paramesenteroides NCFB 803	−	−
Lactobacillus casei ATCC 7469	−	−
Lactobacillus helveticus ATCC 10797	−	−
Lactobacillus bulgaricus ATCC 1489	−	−
Lactobacillus acidophilus ATCC 11842	−	−
Lactobacillus plantarum ATCC 8014	−	−
Lactobacillus fermentum ATCC 9338	−	−

[a] Designation of probes is according to Table 1.

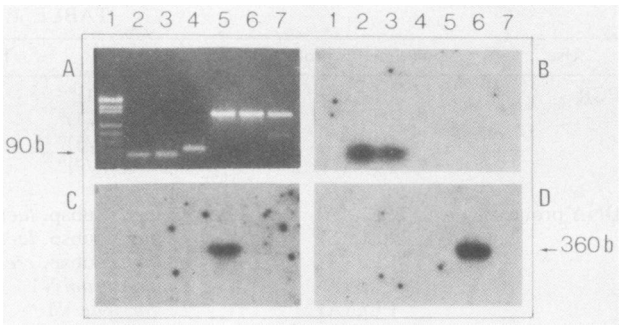

FIG. 3. Identification of *Leuconostoc* species. PCR-amplified DNAs of regions V1 (lane 2 to 4) and V3 (lane 5 to 7) of 16S rRNA genes obtained by using primers P1 + P2 and P3 + P4, respectively, were separated on a 2% agarose gel (A) and stained with ethidium bromide. Lane 1, pUC18 digested with *Hpa*II (19); lanes 2 and 5, *L. mesenteroides* NCFB 523; lanes 3 and 6, *L. lactis* NCFB 533; lanes 4 and 7, *L. paramesenteroides* NCFB 503. Gels run in parallel, which contained identical samples, were blotted and hybridized with the PLC (B), the PLCl (C), or the PLCm (D) probe.

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